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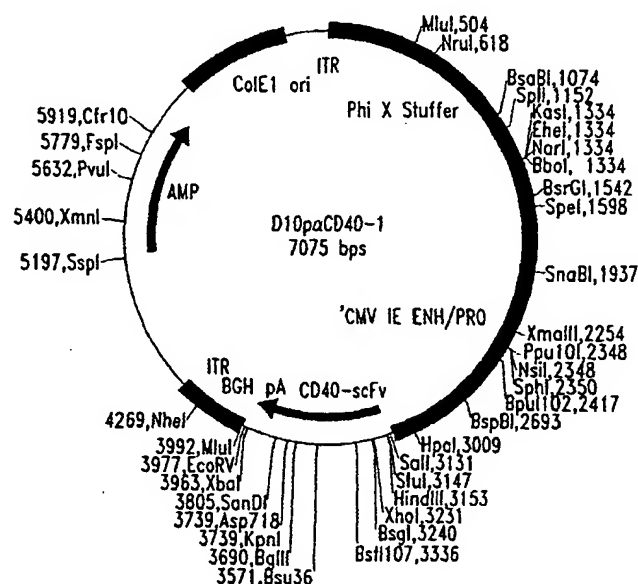
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(54) Title: COMPOSITIONS AND METHODS FOR TREATING AUTOIMMUNE DISEASES AND TRANSPLANT REJECTIONS



(57) Abstract: Gene delivery vectors are used to direct the expression of polynucleotides that encode CD40 antagonists. These CD40 antagonist gene delivery vectors are used to prepare compositions for treating autoimmune diseases and transplant rejections in a mammal. The CD40 antagonist compositions are useful for reversing or substantially diminishing such autoimmune diseases as systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis and psoriasis and for reducing the severity of graft rejection following tissue transplant.

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<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 31025 A (CHIRON CORP) 28 August 1997 (1997-08-28)	1-6
Y	abstract page 1, line 6-10 page 3, line 25 -page 5, line 10 page 6, line 13-24 page 7, line 27 -page 8, line 11 page 8, line 21-25 page 17, line 10 -page 20, line 10 page 27, line 20 -page 30, line 8 -- -/-	7-21
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C.		
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* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "Z" document member of the same patent family		
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Y	abstract page 2, line 5-12 page 7, line 15-31 page 10, line 45 page 12, line 12-17 page 13, line 49-51 page 21, line 14; table 2 page 25, line 5-48 ---	7-23
X	WO 99 42075 A (SQUIBB BRISTOL MYERS CO) 26 August 1999 (1999-08-26)	1-4
Y	abstract  page 4, line 27 -page 5, line 31 page 9, line 26 -page 11, line 2 page 12, line 6-9 page 29, line 2 -page 34, line 19 ---	7-14, 19-23
X	HARTOG DEN M ET AL: "GENERATION OF A HUMANIZED ANTI-CD40 MAB FOR TREATMENT OF IMPORTANCE OF SIZE, SULFATION, AND ANTICOAGULANT ACTIVITY IN THE AUTOIMMUNE DISEASES. POTENTIATION OF ACIDIC FIBROBLAST GROWTH FACTOR BY HEPARIN" IMMUNOTECHNOLOGY,NL,ELSEVIER SCIENCE PUBLISHERS BV, vol. 2, no. 4, 1 November 1996 (1996-11-01), pages 299-689, XP002032468 ISSN: 1380-2933 abstract ---	1,2,5-7, 11-13, 15,17
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Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	PELEGRIN MIREIA ET AL: "Genetically engineered antibodies in gene transfer and gene therapy." HUMAN GENE THERAPY, vol. 9, no. 15, 10 October 1998 (1998-10-10), pages 2165-2175, XP000996554 ISSN: 1043-0342 abstract page 2165, column 1, paragraph 1 -column 2, paragraph 1 page 2166, column 1, paragraph 2	7-23
Y	CROXFORD J LUDOVIC ET AL: "Local gene therapy with CTLA4-immunoglobulin fusion protein in experimental allergic encephalomyelitis." EUROPEAN JOURNAL OF IMMUNOLOGY, vol. 28, no. 12, December 1998 (1998-12), pages 3904-3916, XP000981675 ISSN: 0014-2980 abstract page 3905, column 2, paragraph 1 page 3908, column 1, paragraph 2 page 3913, column 1, paragraph 2	7-23
Y	HAWKINS R E ET AL: "Antibodies: from genes to targeted cancer gene therapy 'editorial!'" GENE THERAPY,GB,MACMILLAN PRESS LTD., BASINGSTOKE, vol. 5, 1998, pages 1581-1583, XP002106269 ISSN: 0969-7128 page 1581, column 1, paragraph 1 -column 2, paragraph 2	7-23
E	WO 01 24823 A (CHIRON CORP) 12 April 2001 (2001-04-12) abstract page 2, line 1-11 page 17, line 26 -page 20, line 19	1-6

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COMPOSITIONS AND METHODS FOR TREATING AUTOIMMUNE DISEASES  
AND TRANSPLANT REJECTIONS

TECHNICAL AREA OF THE INVENTION

This invention relates to compositions and methods for treating  
5 autoimmune diseases and transplant rejections and, more specifically, to the use of gene  
delivery vector technology to direct the expression of CD40 antagonist polynucleotides  
suitable for the treatment of autoimmune diseases and transplant rejections in a  
mammal.

BACKGROUND OF THE INVENTION

10 Conventional antibody based immunosuppressive therapies for treatment  
of autoimmune diseases and transplant rejections require large quantities of antibodies  
for injection. Consequently, such therapeutic regimens can be quite costly and  
inconvenient. Thus, there remains a critical need to identify alternative approaches to  
the administration of antibodies and other molecules for the treatment of autoimmune  
15 diseases and transplant rejections.

SUMMARY OF THE INVENTION

The present invention provides, in one embodiment, polynucleotides that  
encode CD40 antagonists. Inventive CD40 antagonists include, but are not limited to,  
antibodies or fragments thereof as well as fusion proteins, peptides and other molecules  
20 that bind to CD40. Representative CD40 antagonist fusion proteins may comprise at  
least one anti-CD40 antibody Fv heavy chain and one anti-CD40 antibody Fv light  
chain. For example, one such fusion protein comprises portions of the humanized anti-  
CD40 antagonist monoclonal antibody 5H7 Fv heavy and light chains and is encoded  
by the polynucleotide depicted by SEQ ID NO:1 or related polynucleotides having at  
25 least 70% sequence identity with or that hybridize under stringent conditions to the  
polynucleotide of SEQ ID NO:1.

In further embodiments, gene delivery vectors are provided that  
comprise polynucleotides encoding CD40 antagonists. Suitable gene delivery vectors  
for use in expressing inventive CD40 antagonists may comprise transcriptional

sequences from a retrovirus, such as Moloney murine leukemia virus, lentivirus or spumavirus; adenovirus; adeno-associated virus; or herpes virus as well as other viruses suitable for the construction of gene delivery vectors. In one embodiment, the gene delivery vector comprises adeno-associated virus 5' and 3' inverted terminal repeat (ITR) sequences as well as a CMV immediate early enhancer/promoter. The vector can also comprise a signal sequence at the N-terminus, such as a PDGF signal sequence. Additionally, such adeno-associated virus gene delivery vectors may also comprise a polyadenylation sequence for example, the bovine growth hormone (BGH) polyadenylation sequence.

10 All of the gene delivery vectors of the present invention comprise one or more polynucleotides that encode a CD40 antagonist. Representative CD40 antagonists include polypeptides, proteins and other molecules sharing the CD40 antagonist functional activity. For example, the CD40 antagonists may specifically bind to CD40 and may block the interaction of CD40 with its ligand CD40L. Other embodiments of  
15 the present invention provide gene delivery vectors that encode monoclonal or humanized monoclonal antibodies as well as Fab, F(ab)<sub>2</sub> and Fv fragments. Still other embodiments provide gene delivery vectors for the expression of CD40 antagonists derived from the genetic fusion of polynucleotide fragments that encode heavy and light chain Fab and Fv antibody fragments. In one embodiment, the CD40 antagonist is  
20 derived from a cell line that expresses a CD40 monoclonal antibody such as 5H7 or a fragment thereof. In particular, one inventive fusion protein is encoded by the polynucleotide as depicted by SEQ ID NO:1. Other related embodiments provide fusion proteins encoded by polynucleotides that are at least 70% identical with SEQ ID NO:1 or that hybridize under stringent conditions with the polynucleotide depicted in  
25 SEQ ID NO:1.

Additional embodiments of the present invention provide compositions, including pharmaceutical compositions, comprising a therapeutically effective amount of one or more gene delivery vectors that directs the expression of one or more polynucleotides encoding a CD40 antagonist. Inventive compositions may further  
30 comprise a pharmaceutically acceptable carrier or stabilizer suitable for *in vivo* administration. These compositions may be further combined with additional agents efficacious against autoimmune diseases or transplant rejections.

Further embodiments of the present invention provide methods for use of inventive compositions in the treatment of autoimmune diseases in a mammal. By some embodiments, inventive compositions can be formulated in amounts sufficient to reverse or diminish the severity of one or more autoimmune disease such as Hashimoto's thyroiditis, primary myxoedema thyrotoxicosis, pernicious anemia, Addison's disease, insulin-dependent diabetes mellitus, systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis, dermatomyositis, scleroderma and psoriasis.

Other methods embodied within the scope of the present invention include the treatment of transplant rejections by administering compositions that comprise one or more gene delivery vectors encoding a CD40 antagonist. Administration of such compositions by the present methods is expected to permit a diminution or reversal of organ, tissue or cellular transplant rejections.

Inventive compositions may be administered *in vivo* to the mammal through, for example, intravenous, intraperitoneal or intradermal injection. Alternative embodiments provide that the inventive compositions or gene delivery vectors may be administered directly to mammalian cells or tissues *ex vivo* prior to treatment of the mammal with the CD40 antagonist expressing cells or tissue. For example, one or more gene delivery vectors encoding a CD40 antagonist may be applied to peripheral blood mononuclear cells (PBMC) isolated from a subject in need of anti-autoimmune disease therapy prior to reintroducing the PBMC *in vivo*.

Still further embodiments provide *in vitro* methods for the identification of alternative CD40 antagonists including, but not limited to, antibodies and fusion proteins as well as other molecules efficacious in the treatment of autoimmune diseases and transplant rejections.

The present invention thus provides the art with polynucleotides, gene delivery vectors, compositions and methods that are generally effective in treating autoimmune diseases and transplant rejections.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic illustration of the adeno-associated virus based gene delivery vector D10-1.

Figure 2 is a schematic illustration of the adeno-associated virus based gene delivery vector D10p $\alpha$ CD40-1 comprising the coding region of a CD40 antagonist antibody Fv heavy and light chain gene fusion derived from the monoclonal antibody 5H7.

## 5 DETAILED DESCRIPTION OF THE INVENTION

As noted above, the present invention is directed generally to polynucleotides, gene delivery vectors, compositions and methods for the treatment of autoimmune diseases and transplant rejections. More specifically, the invention disclosed herein provides polynucleotides encoding CD40 antagonists, gene delivery  
10 vectors comprising such CD40 antagonist encoding polynucleotides, gene delivery vector based compositions comprising CD40 antagonist encoding polynucleotides and methods for treating autoimmune diseases and transplant rejections by the administration of compositions comprising one or more of these gene delivery vectors and compositions.

15 CD40 is a 40-50 kDa type I membrane glycoprotein belonging to the TNF-R family that is constitutively expressed on B lymphocytes as well as on monocytes, dendritic cells, endothelial cells and epithelial cells. See van Kooten, C. et al., *Int. Arch. Allergy Immunol.* 113:393-399 (1997); Datta, S.K. et al., *Arthritis Rheum* 40(10):1735-45 (1997). The CD40 ligand, referred to variously as CD40L, gp39 or  
20 CD154, is a 33 kDa type II membrane glycoprotein that is transiently expressed primarily on the surface of activated CD4<sup>+</sup> T cells. Datta, *supra*.

Owing to its expression on various cell types of the immune system, CD40 is thought to play a general role in immune regulation. For example, signaling between CD40L and CD40 is believed to be obligatory and nonredundant for helper T-  
25 cell and B-cell interactions leading to T-cell-dependent activation, proliferation or differentiation of B-cells as well as regulation of B-cell survival and apoptosis. See, e.g., Denfeld, R.W. et al., *Eur. J. Immunol.* 26(10):2329-34 (1996). Thus, the CD40-CD40L interaction is likely to be essential in humoral immunity, isotype switching and formation of germinal centers. Support for this hypothesis comes from the observation  
30 that mutations in the CD40L gene cause X-linked hyper-IgM syndrome (X-HIM) which

is characterized by diminished IgG, IgA and IgE serum levels and normal or elevated IgM serum levels. Hill, A. et al., *Nature* 361:494 (1993).

In addition to the role of CD40 and CD40L in immune regulation, these molecules have also been implicated in autoimmune diseases including multiple sclerosis (MS) and systemic lupus erythematosus (SLE) as well as T-cell inflammatory skin diseases such as psoriasis. For example, administration of antibodies to CD40L has been shown to be effective in blocking autoimmunity in proteolipoprotein-induced experimental encephalomyelitis (EAE). As well, EAE is not induced in CD40L knockout mice carrying the T-cell receptor as a transgene. See van Kooten et al., *Int. Arch. Allergy and Immunol.* 113:393-399 (1997) and references cited therein. Similarly, lupus nephritis development in lupus-prone mice is blocked by administration of anti-CD40L antibodies. Further implicating the participation of both CD40 and CD40L in autoimmunity, Cella, M. et al., reported that helper T-cells expressing CD40L and APC expressing CD40 were both detected in brain sections from MS patients. *J. Exp. Med.* 184:747-752 (1996).

It has recently been reported that CD40 and its ligand may play a role in the pathology of psoriasis. Denfeld, *supra*; Wrona-Smith et al., *Am J. Pathol.* 146(5):1079-88 (1995). Psoriasis is a T-cell mediated autoimmune disease believed to be linked to both genetic and environmental triggering factors such as bacterial superantigens. See, e.g., Valdimarsson, H. et al., *Immunol. Today* 16(3):145-9 (1995); Boehncke, W.H. et al., *Nature* 379(6568):777 (29, 1996); Boehncke, W.H., *Trends Microbiol.* 4(12):485-9 (1996). This disease is characterized by complex alterations of various cell types including parakeratosis, the hyperproliferation and differentiation of the epidermal keratinocytes, and acanthosis, the increase in epidermal thickness resulting from keratinocyte hyperproliferation. In addition, psoriatic lesions exhibit an infiltration of mixed leukocytes composed of activated T lymphocytes, neutrophils within the dermis and epidermal microabscesses, lining macrophages and dermal mast cells. Schon, M.P., *J. Invest. Derm.* 112(4):405-410 (1999).

Denfeld, *supra*, observed that human cultured keratinocytes (KC) express CD40 constitutively and that psoriatic KC exhibit elevated CD40 levels. Because CD40 co-localizes with the expression of known markers of inflammatory skin disorders, such as ICAM-1 and Bcl-x, CD40 may have a functional role in the

progression of these diseases, and an effective therapeutic regimen might involve disrupting the CD40/CD40-ligand pathway. *Id.*

In addition to its involvement in autoimmune diseases, CD40 has recently been found to play a critical role in tissue transplant rejection. This observation has significant implications for transplant therapy where the major goal is to induce, in the host mammal, immune tolerance to the donor organ, tissue or cells. That is, successful transplantation requires that a state be induced in the host in which the mammal does not mount an inflammatory response to the allo or xenograft. Gudmundsdottir, H. et al., *J. Am. Soc. Nephrol.* 10:1356-1365 (1999).

It has become well established that T cells require two signals to generate a productive immune response. Janeway, C.A. Jr., et al., *Cell* 76:275-285 (1994); Harding, F.A. et al., *Nature (Lond.)* 356:607-609 (1992); and Jenkins, M.K., et al., *J. Exp. Med.* 165:302-319 (1987). One signal is provided through the T cell receptor (TCR) via the MHC-peptide complex and the second is a costimulatory signal that is necessary for the T cell to respond optimally to antigen. Costimulatory signals amplify and synergize with the TCR ligation.

The costimulatory signal provided through the T cell surface molecule CD28 has been most thoroughly characterized experimentally. CD28 interacts with two ligands, CD80 (B7-1) and CD86 (B7-2). These ligands are expressed on activated antigen presenting cells (APC) and some other cell types. Lenschow, D.J. et al., *Annu. Rev. Immunol.* 14:233-258 (1996) and Reiser, H. et al., *N. Engl. J. Med.* 335:1369-1377 (1996). CD28 costimulation enables T cells to respond to low levels of TCR ligation and supports sustained T cell responses; that is, in the absence of CD28 signaling, T cell responses are transient.

More recently, it has become apparent that CD40 and CD40L play an essential role in T cell costimulation and in transplantation. Thus, it has been reported that CD40 ligation on APC, and other cells, promotes their ability to induce and support T cell responses. For example, engagement of CD40 induces the expression of B7-1. Yang, Y. et al., *Science* 273:1862-1864 (1996). It has, therefore, been suggested that the CD40/CD40L pathway may support T cell immune responses through induction of CD28 ligands and resultant activation of the CD28 pathway. In addition to B7-1, other

molecules important for T cell responses, such as ICAM-1 and CD44H, are also induced in CD40-activated APC. Shinde, S. et al., *J. Immunol.* 157:2764-2768 (1996).

Recent experimental evidence suggests that blockade of T cell co-stimulatory signals may improve long-term allograft survival by inducing transplant tolerance. Blockade of co-stimulatory molecules is effective in both mouse and rat models of cardiac, hepatic, islet, renal, lung and bone marrow transplantation. See, e.g., Sayegh, M.H. et al., *J. Am. Soc. Nephrol.* 6:1143-1150 (1995) and Harlan, D.M. et al., *Graft* 1:63-70 (1998). This was shown, for example, with blocking antibodies to CD40L (CD154). Linsley, P.S. et al., *J. Exp. Med.* 174:561-569 (1991). Hancock, W.H. et al., also examined the role of the CD40-CD40L interaction in transplant rejection and reported that an anti-CD40L monoclonal antibody is effective in minimizing the severity of murine cardiac allograft rejection. *Proc. Natl. Acad. Sci.* 93(24):13967-13972 (1996). Similarly, antibodies to CD40L have been shown to induce transplantation tolerance in murine models of islet transplantation. Larson et al., *Transplantation* 61:4 (1996); Parker et al., *Proc. Natl. Acad. Sci. U.S.A.* 92:9560 (1995).

#### CD40 Antagonists are Effective in the Treatment of Autoimmune Diseases and Transplant Rejections

The present invention provides gene transfer vectors, compositions and methods for treating autoimmune diseases and transplant rejections in a mammal. Each of the inventive gene transfer vectors expresses one or more CD40 antagonist polynucleotide. The present compositions comprise one or more gene transfer vectors having one or more polynucleotide sequence that encodes a CD40 antagonist. Methods encompassed within the scope of the present invention utilize the gene delivery vectors to administer CD40 antagonist polynucleotides to various tissues, organs and cells.

As used herein, the term "antagonist" generally refers to the property of a molecule, compound or other agent to, for example, interfere with the binding of one molecule with another molecule or the stimulation of one cell by another cell either through steric hindrance, conformational alterations or other biochemical mechanism. In one regard, the term antagonist relates to the property of an agent to prevent the binding of a receptor to its ligand, e.g., the binding of CD40 with CD40L, thereby inhibiting the activation of the respective B- or T-cell population. The term antagonist

is not limited to any specific action mechanism, but, rather, refers generally to the functional property presently defined. Antagonists of the present invention include, but are not limited to, antibodies or fragments thereof as well as fusion proteins, peptides and other molecules that bind to CD40.

5           The CD40 antagonists may inhibit the up-regulation of activation markers, *e.g.*, CD25 and CD69, on CD4<sup>+</sup> T-cells to between about 10 and 30% the levels of the untreated control cells. In addition, inventive CD40 antagonists are effective in inhibiting the morphological characteristics of psoriasis such as epidermal thickening and hyperproliferation, *i.e.*, akathosis and parakeratosis, respectively, in the  
10 SCID mouse xenogeneic transplant animal model system.

          The CD40 antagonists described herein can be used to treat other autoimmune diseases characterized by interaction of CD40 with its ligand CD40L. As used herein, the phrase "autoimmune disease" refers generally to those diseases characterized by the failure of one or more B- and/or T-cell populations, or gene  
15 products thereof, to distinguish between self and non-self antigenic determinants. Autoimmune diseases are often characterized by the infiltration of the target cells with inflammatory lymphoid cells, for example, mononuclear phagocytes, lymphocytes and plasma cells as well as secondary lymphoid follicles. Exemplary autoimmune diseases include, but are not limited to, organ specific disorders such as Hashimoto's thyroiditis,  
20 primary myxoedema thyrotoxicosis, pernicious anemia, Addison's disease, and insulin-dependent diabetes mellitus as well as non-organ specific disorders such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), multiple sclerosis, dermatomyositis, scleroderma and psoriasis.

          Similarly, the present CD40 antagonists will also find use in minimizing  
25 or eliminating the onset of graft rejection following tissue transplantation. Thus, CD40 antagonists may be effective in treating, for example, cardiac, hepatic, islet, renal, lung and bone marrow transplantation.

30           1.     *Gene Delivery Vector Systems for the In vivo and  
Ex vivo Expression of CD40 Antagonists*

          As noted above, the present invention provides gene delivery vector systems for the expression of a variety of CD40 antagonists. As used herein, the phrase

“gene delivery vector” refers generally to a nucleic acid construct that carries and, within certain embodiments, is capable of directing the expression of a polynucleotide of interest. For a general overview of gene delivery vector systems, see *Molecular Biotechnology: Principles and Applications of Recombinant DNA*, Ch. 21, pp. 555-590 (ed. B.P. Glick and J.J. Pasternak, 2<sup>nd</sup> ed. 1998); Jolly, *Cancer Gene Ther.* 1:51-64 (1994); Kimura, *Human Gene Ther.* 5:845-852 (1994); Connelly, *Human Gene Ther.* 6:185-193 (1995); and Kaplitt, *Nat. Gen.* 6:148-153 (1994).

A number of virus and non-virus based gene delivery vector systems have been described that are suitable for the administration of CD40 antagonists. Virus based gene delivery systems include, but are not limited to retrovirus, such as Moloney murine leukemia virus, spumaviruses and lentiviruses; adenovirus; adeno-associated virus; and herpes-simplex virus vector systems. Viruses of each type are readily available from depositories or collections such as the American Type Culture Collection (ATCC; Rockville, Maryland) or may be isolated from known sources using commonly available materials and techniques.

The gene delivery vector systems of the present invention will find applications both in *in vivo* as well as *ex vivo* therapeutic regimens. Each of these applications is described in further detail below.

## 2. Retroviral Gene Delivery Vector Systems

Within one aspect of the present invention, retroviral gene delivery vectors are provided which are constructed to carry or express a CD40 antagonist polynucleotide. As used herein, the term “CD40 antagonist polynucleotide” refers generally to a nucleic acid sequence that encodes a polypeptide, protein, fusion protein, antibody, antibody fragment or fusion of antibody fragments having CD40 antagonist activity. Retroviral gene delivery vectors of the present invention may be readily constructed from a wide variety of retroviruses, including for example, B, C, and D type retroviruses as well as spumaviruses and lentiviruses. See *RNA Tumor Viruses*, Cold Spring Harbor Laboratory (2<sup>nd</sup> ed.1985).

Any of the above retroviruses may be readily utilized in order to assemble or construct retroviral gene delivery vectors given the disclosure provided herein, and standard recombinant DNA techniques. See, e.g., Sambrook et al.,

*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press (2d ed. 1989) and Kunkle, *Proc. Natl. Acad. Sci. U.S.A.* 82:488 (1985). In addition, within certain embodiments of the invention, portions of the retroviral gene delivery vectors may be derived from different retroviruses.

5           A retroviral vector, suitable for the expression of a CD40 antagonist polynucleotide, must include at least one transcriptional promoter/enhancer or locus defining element(s), or other elements that control gene expression by other means such as alternate splicing, nuclear RNA export, post-translational modification of messenger, or post-transcriptional modification of protein. Such vector constructs must also  
10 include a packaging signal, long terminal repeats (LTRs) or portion thereof, and positive and negative strand primer binding sites appropriate to the retrovirus used (if these are not already present in the retroviral vector). Optionally, the retroviral vector may also include a signal which directs polyadenylation, selectable markers such as Neomycin resistance, TK, hygromycin resistance, phleomycin resistance histidinol  
15 resistance, or DHFR, as well as one or more restriction sites and a translation termination sequence. Within one aspect of the present invention, retroviral gene delivery vector constructs are provided comprising a 5' LTR, a tRNA binding site, a packaging signal, one or more heterologous sequences, an origin of second strand DNA synthesis and a 3' LTR, wherein the vector construct lacks *gag/pol* or *env* coding  
20 sequences.

Other retroviral gene delivery vectors may likewise be utilized within the context of the present invention, including, for example, those disclosed in the following each of which is incorporated herein by reference: EP 0,415,731; WO 90/07936; WO 94/03622; WO 93/25698; WO 93/25234; U.S. Patent No. 5,219,740;  
25 WO 93/11230; WO 93/10218; Vile et al., *Cancer Res.* 53:3860-3864 (1993); Vile et al., *Cancer Res.* 53:962-967 (1993); Ram et al., *Cancer Res.* 53:83-88 (1993); Takamiya et al., *J. Neurosci. Res.* 33:493-503 (1992); Baba et al., *J. Neurosurg.* 79:729-735 (1993); U.S. Patent No. 4,777,127, GB 2,200,651, EP 0,345,242 and WO 91/02805.

Packaging cell lines suitable for use with the above described retroviral  
30 gene delivery vector constructs may be readily prepared. See, e.g., U.S. Patent Nos. 5,716,832 and 5,591,624. These packaging cell lines may be utilized to create producer cell lines (also termed vector cell lines or "VCLs") for the production of recombinant

vector particles. It may be preferred to use packaging cell lines made from human (*e.g.*, HT1080 cells) or mink parent cell lines, thereby allowing production of recombinant retroviruses that avoid inactivation in human serum.

5           3.     *Adeno-Associated Viral Gene Delivery Vector Systems*

Adeno-associated viruses (AAV) possess a number of qualities that make them particularly suitable for the development of gene delivery vectors generally and for the delivery of polynucleotides encoding CD40 antagonists in particular. For a general review of AAV expression systems, *see* Rabinowitz et al., *Current Opin.*  
10 *Biotech.* 9(5):470-475 (1998). AAV is a non-pathogenic, defective human parvovirus that is non-infective without an adeno or herpes helper virus. Thus, in the absence of a helper virus, AAV becomes integrated latently into the host genome. In addition, AAV has the advantage over the retroviruses, discussed above, in being able to transduce a wide range of both dividing and quiescent cell types.

15           A variety of AAV gene delivery vectors may be utilized to direct the expression of one or more CD40 antagonists. Representative examples of such vectors include the AAV vectors disclosed by Srivastava in WO 93/09239; Samulski et al., *J. Virol.* 63:3822-3828 (1989); Mendelson et al., *Virol.* 166:154-165 (1988); and Flotte et al., *Proc. Natl. Acad. Sci. U.S.A.* 90(22):10613-10617 (1993), incorporated herein by  
20 reference.

Briefly, an AAV gene delivery vector of the present invention should include, in order, a 5' adeno-associated virus inverted terminal repeat; a signal sequence for secretion, such as a PDGF sequence; a polynucleotide encoding VH and VL of the CD40 antagonist wherein the VH and VL regions are separated by a linker; a sequence  
25 operably linked to the CD40 antagonist polynucleotide which regulates its expression in a target tissue, organ or cell; and a 3' adeno-associated virus inverted terminal repeat. A suitable regulatory sequence for the expression of CD40 antagonists is, *e.g.*, the enhancer/promoter sequence of cytomegalovirus (CMV). In addition, the AAV vector may preferably have a polyadenylation sequence such as the bovine growth hormone  
30 (BGH) polyadenylation sequence.

Generally, AAV vectors should have one copy of the AAV ITR at each end of the polynucleotide encoding the CD40 antagonist, to allow replication,

packaging, efficient integration into the host cell genome and rescue from the chromosome. The 5' ITR sequence consists of nucleotides 1 to 145 at the 5' end of the AAV DNA genome, and the 3' ITR includes nucleotides 4681 to 4536 of the AAV genome. Preferably, the AAV vector may also include at least 10 nucleotides following the end of the ITR (*i.e.*, a portion of the so-called "D region").

One exemplary AAV based gene delivery vector encompassed within the scope of the present invention is the D10-1 vector depicted in Figure 1. As discussed in greater detail in Example 1, *infra*, this vector comprises a 145 bp 5' ITR and a 145 bp 3' ITR that flank, in order from 5' to 3', a CMV immediate early (IE) enhancer/promoter (ENH/PRO) sequence for high level transcription of an inserted CD40 antagonist polynucleotide; a multiple cloning site, to permit the convenient subcloning of a desired CD40 antagonist polynucleotide, downstream of the CMV ENH/PRO sequence; a signal sequence; and a polyadenylation (pA) sequence from bovine growth hormone (BGH).

Optimal packaging of an adeno-associated virus gene delivery vector requires that the 5' and 3' ITRs be separated by approximately 2-5 kb. It will be apparent, however, that the ideal spacing between ITR sequences may vary depending on the particular packaging system utilized. This spacing may be achieved by incorporating a "stuffer" or "filler" polynucleotide fragment to bring the total size of the nucleic acid sequence between the two ITRs to between 2 and 5 kb. Thus, where the CD40 antagonist polynucleotide is smaller than 2-5 kb, a non-coding stuffer polynucleotide may be incorporated, for example, 3' to the 5' ITR sequence and 5' of the CD40 antagonist polynucleotide. The precise nucleotide sequence of the stuffer fragment is not an essential element of the final construct. Thus, for example, in the particular AAV based gene delivery vector depicted in Figure 2, the stuffer polynucleotide is a 1.35 kb HaeIII fragment from  $\phi$ X174.

Depending upon the precise application contemplated, rather than incorporating a stuffer fragment, multiple copies of the CD40 antagonist polynucleotide may be inserted, *inter alia*, to achieve the optimal ITR sequence spacing. In such applications, each CD40 antagonist polynucleotide may be separated by a ribosome readthrough or, alternatively, by an Internal Ribosome Entry Site or "IRES." It may also be preferred to incorporate several different CD40 antagonist polynucleotides

separated by a ribosome readthrough or an IRES. In the latter instance, it may be preferred to organize the polynucleotides as two or more separate transcription units each with its own promoter and polyadenylation signal.

Recombinant AAV vectors of the present invention may be generated  
5 from a variety of adeno-associated viruses, including for example, serotypes 1 through 6. For example, ITRs from any AAV serotype are expected to have similar structures and functions with regard to replication, integration, excision and transcriptional mechanisms.

Within certain embodiments of the invention, expression of the CD40  
10 antagonist polynucleotide may be accomplished by a separate promoter (e.g., a viral promoter). Representative examples of suitable promoters in this regard include a CMV promoter, an RSV promoter, an SV40 promoter, or a MoMLV promoter. Other promoters that may similarly be utilized within the context of the present invention include cell or tissue specific promoters or inducible promoters. Representative  
15 inducible promoters include tetracycline-response promoters (e.g., the "Tet" promoter) as described in Gossen et al., *Proc. Natl. Acad. Sci. U.S.A.* 89:5547-5551 (1992); Gossen et al., *Science* 268:1766-1769 (1995); Baron et al., *Nucl. Acids Res.* 25:2723-2729 (1997); Blau et al., *Proc. Natl. Acad. Sci. U.S.A.* 96:797-799 (1999); Bohl et al., *Blood* 92:1512-1517 (1998); and Haberman et al., *Gene Therapy* 5:1604-1611 (1998);  
20 the ecdysone promoter system as described in No et al., *Proc. Natl. Acad. Sci. U.S.A.* 93:3346-3351 (1996); and other regulated promoters or promoter systems as described in Rivera et al., *Nat. Med.* 2:1028-1032 (1996).

The AAV gene delivery vector may also contain additional sequences, for example from an adenovirus, which assist in effecting a desired function for the  
25 vector. Such sequences include, for example, those which assist in packaging the AAV gene delivery vector in adenovirus particles.

Packaging cell lines suitable for producing adeno-associated viral vectors may be routinely prepared given readily available techniques. See, e.g., U.S. Patent No. 5,872,005, incorporated herein by reference. At a minimum, suitable  
30 packaging systems for AAV gene delivery systems of the present invention will include the AAV replication and capsid genes.

Preferred packaging cell lines may contain both an AAV helper virus as well as an AAV gene delivery vector containing the CD40 antagonist polynucleotide. For detailed descriptions of representative packaging cell line systems, *see, e.g.* Holscher, C. et al., *J. Virol.* 68:7169-7177 (1994); Clark, K.R. et al., *Hum. Gene Ther.* 6:1329-1341 (1995); and Tamayosa, K. et al., *Hum. Gen. Ther.* 7:507-513 (1996) which are incorporated herein by reference.

Alternatively, packaging of AAV may be achieved *in vitro* in a cell free system to obviate transfection protocols or packaging cell lines. Such *in vitro* systems incorporate an AAV gene delivery vector bearing the CD40 antagonist polynucleotide and a source of Rep-protein, capsid-protein and Adenovirus proteins that supply helper-viral functions. The latter proteins are typically supplied in the form of a cell extract. Representative *in vitro* systems are further described in Ding, L. et al., *Gen. Ther.* 4:1167-1172 (1997) and Zhou, Z. et al., *J. Virol.* 72:3241-3247 (1998) which are incorporated herein by reference.

#### 4. Other Viral Gene Delivery Vector Systems

In addition to retroviral vectors and adeno-associated virus-based vectors, numerous other viral gene delivery vector systems may also be utilized for the expression of CD40 antagonists. For example, within one embodiment of the invention adenoviral vectors may be employed. Representative examples of such vectors include those described by, for example, Berkner, *Biotechniques* 6:616-627 (1988); Rosenfeld et al., *Science* 252:431-434 (1991); WO 93/9191; Kolls et al., *Proc. Natl. Acad. Sci. U.S.A.* 91(1):215-219 (1994); Kass-Eisler et al., *Proc. Natl. Acad. Sci. U.S.A.* 90(24):11498-502 (1993); Guzman et al., *Circulation* 88(6):2838-48 (1993); Guzman et al., *Cir. Res.* 73(6):1202-1207 (1993); Zabner et al., *Cell* 75(2):207-216 (1993); Li et al., *Hum. Gene Ther.* 4(4):403-409 (1993); Caillaud et al., *Eur. J. Neurosci.* 5(10):1287-1291 (1993); Vincent et al., *Nat. Genet.* 5(2):130-134 (1993); Jaffe et al., *Nat. Genet.* 1(5):372-378 (1992); and Levrero et al., *Gene* 101(2):195-202 (1991); and WO 93/07283; WO 93/06223; and WO 93/07282.

Gene delivery vectors of the present invention also include herpes vectors. Representative examples of such vectors include those disclosed by Kit in *Adv. Exp. Med. Biol.* 215:219-236 (1989); and those disclosed in U.S. Patent No. 5,288,641

and EP 0176170 (Roizman). Additional exemplary herpes simplex virus vectors include HFEM/ICP6-LacZ disclosed in WO 95/04139 (Wistar Institute), pHSVlac described in Geller, *Science* 241:1667-1669 (1988), and in WO 90/09441 and WO 92/07945; HSV Us3::pgC-lacZ described in Fink, *Human Gene Therapy* 3:11-19  
5 (1992); and HSV 7134, 2 RH 105 and GAL4 described in EP 0453242 (Breakefield), and those deposited with the ATCC as accession numbers ATCC VR-977 and ATCC VR-260.

Gene delivery vectors may also be generated from a wide variety of other viruses including, for example, poliovirus (Evans et al., *Nature* 339:385-388  
10 (1989); and Sabin, *J. Biol. Standardization* 1:115-118 (1973)); rhinovirus; pox viruses, such as canary pox virus or vaccinia virus (Fisher-Hoch et al., *Proc. Natl. Acad. Sci. U.S.A.* 86:317-321 (1989); Flexner et al., *Ann. N.Y. Acad. Sci.* 569:86-103 (1989); Flexner et al., *Vaccine* 8:17-21 (1990); U.S. Patent Nos. 4,603,112, 4,769,330 and 5,017,487; WO 89/01973); SV40 (Mulligan et al., *Nature* 277:108-114 (1979);  
15 influenza virus (Luytjes et al., *Cell* 59:1107-1113 (1989); McMichael et al., *N. Eng. J. Med.* 309:13-17 (1983); and Yap et al., *Nature* 273:238-239 (1978)); HIV (Poznansky, *J. Virol.* 65:532-536 (1991)); measles (EP 0 440,219); astrovirus (Munroe et al., *J. Vir.* 67:3611-3614 (1993)); and coronavirus, as well as other viral systems (e.g., EP 0,440,219; WO 92/06693; U.S. Patent No. 5,166,057).

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#### 5. Non-viral Gene Delivery Vectors

Other gene delivery vectors and methods that may be employed for the expression of CD40 antagonists such as, for example, nucleic acid expression vectors; polycationic condensed DNA linked or unlinked to killed adenovirus alone, for  
25 example, see Curiel, *Hum Gene Ther* 3:147-154 (1992); ligand linked DNA, for example, see Wu, *J Biol Chem* 264:16985-16987 (1989); eucaryotic cell delivery vectors; deposition of photopolymerized hydrogel materials; hand-held gene transfer particle gun, as described in U.S. Patent No. 5,149,655; ionizing radiation as described in U.S. 5,206,152 and in WO 92/11033; nucleic charge neutralization or fusion with cell  
30 membranes. Additional approaches are described in Philip, *Mol Cell Biol* 14:2411-2418 (1994), and in Woffendin, *Proc. Natl. Acad. Sci.* 91:1581-1585 (1994).

Particle mediated gene transfer may be employed. Briefly, the CD40 antagonist polynucleotide of interest can be inserted into conventional vectors that contain conventional control sequences for high level expression, and then incubated with synthetic gene transfer molecules such as polymeric DNA-binding cations like polylysine, protamine, and albumin, linked to cell targeting ligands such as asialoorosomucoid, as described in Wu, et al., *J. Biol. Chem.* 262:4429-4432 (1987), insulin as described in Hucked, *Biochem Pharmacol* 40:253-263 (1990), galactose as described in Plank, *Bioconjugate Chem* 3:533-539 (1992), lactose or transferrin.

Naked DNA may also be employed. Exemplary naked DNA introduction methods are described in WO 90/11092 and U.S. Patent No. 5,580,859. Uptake efficiency may be improved using biodegradable latex beads. DNA coated latex beads are efficiently transported into cells after endocytosis initiation by the beads. The method may be improved further by treatment of the beads to increase hydrophobicity and thereby facilitate disruption of the endosome and release of the DNA into the cytoplasm.

Liposomes that can act as gene delivery vehicles are described in U.S. Patent No. 5,422,120, PCT Patent Publication Nos. WO 95/13796, WO 94/23697, and WO 91/144445, and European Patent Publication No. 524,968. Nucleic acid sequences can be inserted into conventional vectors that contain conventional control sequences for high level expression, and then incubated with synthetic gene transfer molecules such as polymeric DNA-binding cations like polylysine, protamine, and albumin, linked to cell targeting ligands such as asialoorosomucoid, insulin, galactose, lactose, or transferrin. Other delivery systems include the use of liposomes to encapsulate DNA comprising the gene under the control of a variety of tissue-specific or ubiquitously-active promoters. Further non-viral delivery suitable for use includes mechanical delivery systems such as the approach described in Woffendin et al., *Proc. Natl. Acad. Sci. U.S.A.* 91(24):11581-11585 (1994). Moreover, the coding sequence and the product of expression of such can be delivered through deposition of photopolymerized hydrogel materials.

Exemplary liposome and polycationic gene delivery vehicles are those described in U.S. Patent Nos. 5,422,120 and 4,762,915, in PCT Patent Publication Nos. WO 95/13796, WO 94/23697, and WO 91/14445, in European Patent Publication No.

524,968 and in Starrier, *Biochemistry*, pp. 236-240 (1975) W.H. Freeman, San Francisco; Shokai, *Biochem. Biophys. Acta.* 600:1 (1980); Bayer, *Biochem. Biophys. Acta.* 550:464 (1979); Rivet, *Methods Enzymol.* 149:119 (1987); Wang, *Proc. Natl. Acad. Sci. U.S.A.* 84:7851 (1987); Plant, *Anal. Biochem.* 176:420 (1989).

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a. *Identification of CD40 Antagonists and Isolation of the Corresponding Polynucleotides*

As noted above, the present invention provides compositions and methods for treating autoimmune diseases and transplant rejections by the administration of gene delivery vectors comprising CD40 antagonist polynucleotides. CD40 antagonists within the scope of the present invention include, but are not limited to antibodies, or fragments thereof, as well as fusion proteins or other molecules that bear the CD40 antagonist functional activity.

In one embodiment of the present invention, CD40 antagonists are monoclonal antibodies prepared essentially as described in de Boer et al. U.S. Patent No. 5,677,165 (1997) (de Boer '165) which patent is incorporated herein by reference. By this method, DNA encoding CD40 or a fragment thereof is PCR amplified from a mixture of cellular cDNAs. The PCR product is digested with one or more restriction endonucleases to create appropriate ends and ligated into a baculovirus plasmid or other expression system. In the case of a baculovirus expression system, the plasmid encoding CD40, or a fragment thereof, is introduced into, e.g., Sf9 cells to facilitate protein production. Clones of Sf9 cells expressing CD40 are identified, e.g., by enzyme linked immunosorbant assay (ELISA) as discussed in de Boer '165 and injected, intraperitoneally, into BALB/c mice to induce antibody production. Serum is tested for the production of specific antibodies and spleen cells from animals having a positive specific antibody titer are used for cell fusions with myeloma cells to generate hybridoma clones. Supernatants derived from hybridoma clones are tested, via fluorescent cell staining of EBV-transformed B-cells, for the presence of monoclonal antibodies having specificity against CD40.

In other embodiments of the present invention, CD40 antagonists are humanized anti-CD40 monoclonal antibodies. The phrase "humanized antibody" refers to an antibody derived from a non-human antibody - typically a mouse monoclonal

antibody. Alternatively, a humanized antibody may be derived from a chimeric antibody that retains or substantially retains the antigen-binding properties of the parental, non-human, antibody but which exhibits diminished immunogenicity as compared to the parental antibody when administered to humans. The phrase "chimeric antibody," as used herein, refers to an antibody containing sequences derived from two different antibodies (*see, e.g.*, U.S. Patent No. 4,816,567), which typically originate from different species. Most typically, chimeric antibodies comprise human and murine antibody fragments, generally human constant and mouse variable regions.

Because humanized antibodies are far less immunogenic in humans than the parental mouse monoclonal antibodies, they can be used for the treatment of humans with far less risk of anaphylaxis. Thus, these antibodies may be preferred in therapeutic applications that involve *in vivo* administration to a human afflicted with an autoimmune disease or, alternatively, experiencing, or at risk of experiencing, a transplant rejection.

Another approach for the isolation of fully humanized anti-CD40 antibodies is afforded by the so-called xenomouse technology. *See* Jakobovits et al., *Adv. Drug Deliv. Rev.* 31:33-42 (1998); Jakobovits et al., *Exp. Opin. Invest. Drugs* 7(4):607-614 (1998); Mendez et al., *Nature Genet.* 15:146-156 (1997); Jakobovits et al., *Cur. Opin. Biotech.* 6(5):561-566 (1995); Green, L.L., et al., *Nature Genet.* 7:13-21 (1994); and Lonberg, N. et al., *Nature* 368:856-859 (1994) each of which is incorporated herein by reference. By this methodology, mice are genetically engineered to suppress mouse antibody gene expression. The mouse antibody repertoire is functionally replaced with human antibody gene expression, while the rest of the mouse immune system is left intact. These xenomice are capable of generating human antibodies to human antigens, such as CD40, which antigens are recognized as foreign by the mouse such that an immune response is elicited.

Generation of humanized anti-CD40 antibodies by the xenomouse technology requires the inoculation of mice with either soluble or cell-surface expressed CD40 protein. CD40 suitable for inoculation may be prepared by recombinant DNA methods as discussed, *supra*, and as otherwise well known in the art. *See, e.g.*, Sambrook et al, *supra* and de Boer '165, *supra*. A strong CD40 antigen-specific antibody response may be detected by ELISAs performed on sera from immunized mice. Hybridomas may

be derived from spleen or lymph node by standard hybridoma technology and screened for secretion of antigen-specific human monoclonal antibodies by ELISA. See, e.g., Harlow and Lane, *Antibodies: A Laboratory Manual*, supra for a discussion of the hybridoma technology.

5           Examples of suitable human anti-CD40 monoclonal antibodies are disclosed in applicants' co-pending application entitled "Human Anti-CD40 Monoclonal Antibodies," filed October 2, 2000, as Serial No. \_\_\_\_\_, which is incorporated herein by reference. The application discloses CD40 antibodies raised in mice transgenic for human immunoglobulin loci. The antibodies specifically bind to  
10 CD40 expressed by a variety of cells, and are suitable for use in the methods of the present invention.

Humanized antibodies may be achieved by a variety of methods including, for example: (1) grafting the non-human complementarity determining regions (CDRs) onto a human framework and constant region (a process referred to in  
15 the art as "humanizing"), or, alternatively, (2) transplanting the entire non-human variable domains, but "cloaking" them with a human-like surface by replacement of surface residues (a process referred to in the art as "veneering"). In the present invention, humanized antibodies will include both "humanized" and "veneered" antibodies. These methods are disclosed in, e.g., Jones et al., *Nature* 321:522-525  
20 (1986); Morrison et al., *Proc. Natl. Acad. Sci., U.S.A.* 81:6851-6855 (1984); Morrison and Oi, *Adv. Immunol.* 44:65-92 (1988); Verhoeyer et al., *Science* 239:1534-1536 (1988); Padlan, *Molec. Immunol.* 28:489-498 (1991); Padlan, *Molec. Immunol.* 31(3):169-217 (1994); and Kettleborough, C.A. et al., *Protein Eng.* 4(7):773-83 (1991) each of which is incorporated herein by reference.

25           Hoogenboom, H., et al., U.S. Patent No. 5,565,332; Winter, G. and Milstein, C., *Nature* 349:293-299 (1991); Riechmann et al., *Nature* 332:323-327 (1988); Hird et al., *Br. J. Cancer* 64:911-914 (1991); Gassow and Seemann, *Methods in Enzymology*, pp 99-121 (1991); Jones et al., *Nature* 321:522-525 (1986); Verhoeyen et al., *Science* 239:1534-1536 (1988); Kamman et al., *Nucl. Acids Res* 17:5404 (1989);  
30 Maeda et al., *Hum. Antibod. Hybridomas* 2:124-133 (1991) which are incorporated herein by reference, also describe humanized antibodies. By this method, the complementarity determining regions (CDRs) of a mouse antibody are inserted into

human framework regions to generate antibodies in which only the CDR sequences are derived from the original mouse antibody. There are three CDRs in the heavy chains and three in the light chains that comprise the antibody's antigen binding region. The CDRs are identified by comparing the amino acid sequence of the variable regions of the mouse monoclonal antibody to the sequences of all known mouse variable regions, and the sequences of all known human variable regions. See Kabat et al., *Sequences of Proteins of Immunological Interest*, U.S. Department of Health and Human Services, U.S. Government Printing Office, Washington, D.C. (4th ed. 1987).

PCR methodology may be used to graft CDRs from mouse, or other rodent, monoclonal antibodies onto human FRs to make fully humanized antibodies or antibody fragments. Lewis, A.P. and Crowe, J.S., *Gene* 101(2):297-302 (1991). PCR primers that bind to the FR regions flanking the CDRs have been developed; these permit amplification of the CDR from hybridoma cells secreting the CD40 antagonist monoclonal antibody of interest. The PCR product is cloned and sequenced, and joined to the framework and constant regions of choice.

The phrase "complementarity determining region" refers to amino acid sequences which together define the binding affinity and specificity of the natural Fv region of a native immunoglobulin binding site. See, e.g., Chothia et al., *J. Mol. Biol.* 196:901-917 (1987); Kabat et al., U.S. Dept. of Health and Human Services NIH Publication No. 91-3242 (1991). The phrase "constant region" refers to the portion of the antibody molecule which confers effector functions. In the present invention, mouse constant regions are substituted by human constant regions. The constant regions of the subject humanized antibodies are derived from human immunoglobulins. The heavy chain constant region can be selected from any of the five isotypes: alpha, delta, epsilon, gamma or mu.

One method of humanizing antibodies comprises aligning the non-human heavy and light chain sequences to human heavy and light chain sequences, selecting and replacing the non-human framework with a human framework based on such alignment, molecular modeling to predict the conformation of the humanized sequence and comparing to the conformation of the parent antibody. This process is followed by repeated back mutation of residues in the CDR region which disturb the structure of the CDRs until the predicted conformation of the humanized sequence

model closely approximates the conformation of the non-human CDRs of the parent non-human antibody. Such humanized antibodies may be further derivatized to facilitate uptake and clearance, *e.g.*, via Ashwell receptors. *See, e.g.*, U.S. Patent Nos. 5,530,101 and 5,585,089 which patents are incorporated herein by reference.

5           Alternatively, humanized antibodies may be prepared essentially as described in de Boer, U.S. Patent No. 5,874,082 (1999) (de Boer '082) which patent is incorporated herein by reference. Briefly, mRNA is prepared from a hybridoma which expresses an anti-CD40 monoclonal antibody. cDNA encoding the variable regions of the heavy and light chains is amplified using RT-PCR employing degenerate  
10   oligonucleotide primers. As disclosed in de Boer '082, the RT-PCR technique is well known in the art and is incorporated herein by reference to Myers et al., *Biochemistry*, 30:7661-7666 (1991) and U.S. Patent Nos. 5,310,652 and 5,407,800. PCR products are cloned into a sequencing plasmid from which clones the nucleotide sequence of the variable heavy and light chain cDNAs are determined and from which sequence a  
15   consensus amino acid sequence for the variable heavy and light chains is derived.

          The deduced amino acid sequences are used to search databases for human antibody sequences having the highest degree of sequence similarity to the monoclonal antibody (de Boer '082). Based on the identified homologous human sequence, mutagenesis primers are designed and used to change the indicated residues  
20   from mouse to human. cDNAs encoding the humanized variable heavy and light chains are expressed off a baculovirus expression plasmid including a portion of the constant region of human IgG heavy chain and the complete human constant light chain. Humanized heavy and light chains are co-expressed in Sf9 insect cells and the resulting culture supernatants are analyzed for antibody expression using Western blot and  
25   fluorescence-activated cell sorting (FACS) analysis (de Boer '082).

          It will be appreciated that alternative CD40 antagonists may be readily obtained by other methods commonly known in the art. Because of the desirability of having fully humanized sequences, depending on the precise application involved, any of the following methods may be preferred. For example, CD40 antagonists within the  
30   scope of the present invention may be isolated by screening Fab phage display libraries using recombinant CD40 as a probe. Also, CD40 antagonist antibodies may be derived from hybridoma cells produced from spleen cells of one or more xenomouse inoculated

with CD40. See, e.g., Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory (1988) for a discussion of the hybridoma technology. Another methodology that may be employed to obtain humanized CD40 antagonists suitable for expression in a gene delivery vector of the present invention is the CDR grafting technology. These methods are described in further detail below.

Phage display libraries for the production of high-affinity antibodies have been the subject of several recent reviews. See, e.g., Hoogenboom, H.R. et al., *Immunotechnology* 4(1):1-20 (1998); Hoogenboom, H.R., *Trends Biotechnol.* 15:62-70 (1997) and McGuinness, B. et al., *Nature Bio. Technol.* 14:1149-1154 (1996) each of which is incorporated herein by reference. Among the advantages of the phage display technology is the ability to isolate antibodies of human origin that cannot otherwise be easily isolated by conventional hybridoma technology. Furthermore, phage display antibodies may be isolated *in vitro* without relying on an animal's immune system.

Antibody phage display libraries may be accomplished, for example, by the method of McCafferty et al., *Nature* 348:552-554 (1990) which is incorporated herein by reference. In short, the coding sequence of the antibody variable region is fused to the amino terminus of a phage minor coat protein (pIII). Expression of the antibody variable region-pIII fusion construct results in the antibody's "display" on the phage surface with the corresponding genetic material encompassed within the phage particle.

CD40 suitable for screening a phage library may be expressed on baculovirus Sf9 cells as detailed in deBoer '165, *see supra*. Alternatively, the CD40 coding region may be PCR amplified using primers specific to the CD40 extracellular domain. The resulting fragment may then be isolated by, e.g., agarose gel purification and ligated in frame with a suitable tag protein such as 6-His, glutathione-S-transferase (GST) or other such readily available affinity tag. See, e.g., *Molecular Biotechnology: Principles and Applications of Recombinant DNA*, *supra*, pp. 160-161.

The resulting fusion protein may then be adsorbed to a solid matrix, e.g., a tissue culture plate or bead. Phage expressing antibodies having the desired anti-CD40 binding properties may subsequently be isolated by successive panning, in the case of a solid matrix, or by affinity adsorption to a CD40 antigen column. Phage having the desired CD40 antagonist properties may be reintroduced into bacteria by

infection and propagated by standard methods known to those skilled in the art. *See* Hoogenboom, H.R., *Trends Biotechnol.*, *supra* for a review of methods for screening for positive antibody-pIII phage.

Identification of additional CD40 antagonists may be achieved by using  
5 any of a number of known methods for identifying and obtaining proteins that specifically interact with other proteins or polypeptides, for example, a yeast two-hybrid screening system such as that described in U.S. Patent No. 5,283,173 and U.S. Patent No., 5,468,614. In one embodiment of the present invention, a cDNA encoding CD40, or a fragment thereof, may be cloned into a two-hybrid bait vector and used to  
10 screen a complementary target library for a protein having CD40 binding activity.

Polynucleotides that encode antibodies, antibody fragments or peptides having CD40 antagonist activity may be readily obtained by conventional recombinant DNA methodology. *See, e.g.*, Sambrook et al., *supra*. For example, RNA may be isolated from one or more hybridoma cell lines derived from spleen cells of immunized  
15 animals that express CD40 antagonist antibodies and the respective polynucleotide encoding that antibody or antibody Fab heavy and light chains may be isolated, *e.g.*, by the polymerase chain reaction (PCR) using oligonucleotides specific to the CD40 antagonist antibody sequence. The CD40 antagonists of the present invention are said to be immunospecific or specifically binding if they bind to CD40 with a  $K_a$  of greater  
20 than or equal to about  $10^4 \text{ M}^{-1}$ , preferably of greater than or equal to about  $10^5 \text{ M}^{-1}$ , more preferably of greater than or equal to about  $10^6 \text{ M}^{-1}$  and still more preferably of greater than or equal to about  $10^7 \text{ M}^{-1}$ . Such affinities may be readily determined using conventional techniques, such as by equilibrium dialysis; by using the BIAcore 2000 instrument, using general procedures outlined by the manufacturer; by  
25 radioimmunoassay using  $^{125}\text{I}$ -labeled CD40; or by another method known to the skilled artisan. The affinity data may be analyzed, for example, by the method of Scatchard et al., *Ann N.Y. Acad. Sci.*, 51:660 (1949). Thus, it will be apparent that preferred CD40 antagonists will exhibit a high degree of specificity for CD40 and will bind with substantially lower affinity to other molecules.

30 CD40 antagonists of the present invention include, where applicable, functional equivalents. For example, molecules may differ in length, structure, components, etc. but may still retain one or more of the defined functions. More

particularly, functional equivalents of the antibodies, antibody fragments or peptides of the present invention may include mimetic compounds, *i.e.*, constructs designed to mimic the proper configuration and/or orientation for antigen binding.

Preferred CD40 antagonists may comprise one or more conservative amino acid substitutions. By "conservative amino acid substitutions" is meant those changes in amino acid sequence that preserve the general charge, hydrophobicity/hydrophilicity and/or steric bulk of the amino acid substituted. For example, substitutions between the following groups are conservative: Gly/Ala, Val/Ile/Leu, Asp/Glu, Lys/Arg, Asn/Gln, Ser/Cys/Thr, and Phe/Trp/Tyr. Such modifications will not substantially diminish the efficacy of the CD40 antagonists and may impart such desired properties as, for example, increased *in vivo* half-life or decreased toxicity.

A CD40 Antagonist Single Chain Antibody  
Derived from the Monoclonal Antibody 5H7

The present invention provides, in certain embodiments, single-chain antibody fusion proteins comprising one or more Fv heavy chain and one or more Fv light chain. Single-chain antibodies may be used for a variety of therapeutic and diagnostic applications in which Fc effector functions are not required and when a small size is advantageous. It may also be desirable to link additional protein-coding sequences onto CD40 antagonist polynucleotides to create dual-function molecules that can bind to CD40 and carry with the CD40 antagonist an independent functional activity.

An exemplary CD40 antagonist is depicted by the amino acid sequence of SEQ ID NO:2 which is the protein product of the polynucleotide of SEQ ID NO:1. As discussed in detail below, *see* Example 1, *infra*, this single-chain anti-CD40 antagonist antibody was derived from the hybridoma 5H7 cell line by fusion of the coding region of an anti-CD40 antagonist Fv heavy chain gene and an anti-CD40 antagonist Fv light chain gene. More specifically, RNA was isolated from the 5H7 hybridoma cell line, amplified by reverse transcriptase polymerase chain reaction (RT-PCR) using two pairs of oligonucleotide primers specific to the Fab heavy and light chain sequences, the resulting DNA fragments were connected by a linker sequence and

were ligated into the AAV-derived gene delivery vector D10-1, Figure 1, to create the vector D10p $\alpha$ CD40-1, Figure 2.

It will be understood that the orientation of the VH and VL chains may be reversed or otherwise fused to alternative heavy and/or light chain sequences to create CD40 antagonist molecules having the desired functional properties. Also, the length and nucleotide sequence of the linker region may be varied as necessary in order to achieve optimal CD40 binding affinity or other property appropriate for a given therapeutic or diagnostic application.

It will also be apparent that the nucleotide sequence of SEQ ID NO:1 may be varied to modify the CD40 antagonist's biological properties, *in vivo* stability or other functionality in accordance with the particular application desired. Thus, the present invention contemplates modifications of the sequence of SEQ ID NO:1 wherein the resulting polynucleotide is not less than 70% identical with the nucleic acid sequence of SEQ ID NO:1. Preferably, the resulting polynucleotide is not less than 90% identical with the nucleic acid sequence of SEQ ID NO:1. More preferably, the resulting polynucleotide is not less than 98% identical with the nucleic acid sequence of SEQ ID NO:1.

Such amino acid sequence identity may be determined by standard methodologies, including use of the National Center for Biotechnology Information BLAST search methodology available at [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov) using default parameters. The identity methodologies most preferred are those described in U.S. Patent 5,691,179 and Altschul et al., *Nucleic Acids Res.* 25:3389-3402 (1997) both of which are incorporated herein by reference. Global DNA sequence identity may also be determined using the Smith-Waterman homology search algorithm as implemented in MPSRCH program (Oxford Molecular) using an affine gap search with the following search parameters: gap open penalty: 12, gap extension penalty: 1.

The present invention also contemplates that polynucleotides comprising a portion or portions of the nucleic acid sequence of SEQ ID NO:1 may provide the desired CD40 antagonist activity. Thus, the present invention provides polynucleotides or the complements thereof that hybridize under stringent conditions to the polynucleotide depicted by SEQ ID NO:1.

As will be recognized by those skilled in the art, to "hybridize" under conditions of a specified stringency is commonly used to describe the stability of hybrids formed between two single-stranded nucleic acid molecules. Stringency of hybridization is typically expressed in conditions of ionic strength and temperature at which such hybrids are annealed and washed. Typically, high, medium and low stringency encompass the following conditions or equivalent conditions thereto: (1) By high stringency is meant 0.1 x SSPE or SSC, 0.1% SDS, 65°C; (2) by medium stringency is meant 0.2 x SSPE or SSC, 0.1% SDS, 50°C; and (3) by low stringency is meant 1.0 x SSPE or SSC, 0.1% SDS, 50°C.

10

Animal Model Systems Suitable for Confirming the Efficacy CD40 Antagonists  
Expressed from Gene Delivery Vectors

Compounds potentially useful in treating autoimmune diseases and transplant rejections may be screened in a number of systems. Animal models are used to identify those compounds having therapeutic activity *in vivo* as well as possessing acceptable levels of host toxicity. The models are useful for identifying compounds that are efficacious in the treatment of autoimmune diseases such as, *e.g.*, systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis and psoriasis as well as against transplant rejections, for example, those occurring after transplantation of cardiac, hepatic, islet, renal, lung and bone marrow tissue.

20

Efficacy of a given CD40 antagonist gene delivery vector can be tested in any of the animal model systems familiar to those skilled in the art. Animal model systems for autoimmune diseases are described in Roitt, I. et al., "Autoimmunity and Autoimmune Disease," *Immunology*, Ch. 28 (1998); animal model systems available for the study of psoriasis, in particular, are described in Schon, M.P., *supra*. The skilled artisan will appreciate that the selection of an appropriate animal model system will depend on the particular disease being treated. The following animal model systems are, therefore, provided by way of example not limitation.

25

It is well known in the art that autoimmunity can be induced in experimental animals by injecting autoantigen (*i.e.*, self antigen) together with Freund's adjuvant. Thus, such an animal model system may be used, for example, by injecting thyroglobulin to induce an inflammatory disease of the thyroid. With such a model

30

system, not only are thyroid autoantibodies produced, but, also, the gland becomes infiltrated with mononuclear cells and the acinar architecture deteriorates. This animal model has been used to model the human condition known as Hashimoto's thyroiditis. In a similar fashion, myelin basic protein, or T-cells specific for myelin basic protein, may be injected in mice or rats to induce autoallergic encephalomyelitis.

Alternative animal model systems that may be used to test compounds and treatment regimens within the scope of the present invention include animals exhibiting spontaneous autoimmune diseases. By way of example and not limitation, the Obese strain (OS) of chicken is characterized by the spontaneous occurrence of autoantibodies and by the progressive destruction and chronic inflammation of the thyroid. The OS chicken parallels human autoimmune thyroid disease in displaying thyroid lesions as well as the production of antibodies to various thyroid components.

A number of animal model systems for psoriasis have been described including transplantation of human psoriatic skin onto nude mice, the asebia (*ab/ab*) strain of mice or the HLA-B27 transgenic rat as well as transplantation of skin from the flaky skin mouse onto nude mice. Nickoloff, B.J. et al., *Am. J. Path.*, 146(3):580-588 (1995); Schon, *supra*. The asebia mouse model features epidermal akantosis, increased dermal vascularity and dermal infiltrate of macrophages and mast cells, but does not contain T-cell and neutrophil infiltrates. Nickoloff, *supra*. Thus, the skin alterations in the *ab/ab* mouse do not precisely mirror every biological characteristic of psoriatic lesions.

In addition to the above mentioned animal model systems, the SCID mouse is widely used as an *in vivo* model of psoriasis. *Id.* A standard measure of efficacy in the SCID model is the ability to lessen the severity of akantosis and parakeratosis as well as to reduce mononuclear cell infiltrate in animals transplanted with psoriatic skin.

In recent years, it has been observed that human skin cells can be engrafted onto severe combined immunodeficiency (SCID) mice with long-term graft survival. The SCID mouse is also amenable to the adoptive transfer of components of the human immune system. See, e.g., Boehncke, W.-H. et al., *Arch. Dermatol. Res.*, 286:325-330 (1994). The autosomal recessive mutation responsible for the SCID phenotype in mice prevents antigen receptor gene rearrangements resulting in an

intrinsic defect of T- and B-cells. Botsma, M.J. et al., *Annu. Rev. Immunol.*, 9:323-350 (1991). Nickoloff, *supra*, reported that psoriatic plaque skin (PP), normal human skin from healthy individuals (NN) and symptomless skin from a patient with psoriasis (PN) can be transplanted onto SCID mice with retention of clinical, histological and immunological phenotypic characteristics.

The various animal models for psoriasis have recently been the subject of review by M.P. Schon, *supra*. Significantly, Schon reported that the SCID mouse xenogeneic skin transplant model system exhibits the morphological and pathological characteristics of naturally occurring human psoriasis. For example, psoriatic human skin transplanted onto the SCID mouse maintains the psoriatic phenotype as evidenced by akathosis and hyperproliferation. Also, transplanted skin is characterized by altered keratinocyte differentiation, induction of MHC Class II and ICAM-1, increased vascularity, T-cell and neutrophil infiltrate and intraepidermal microabscesses. Thus, Schon endorses the SCID mouse for studies of antipsoriatic treatments noting, in particular, that the attractiveness of this animal model stems from its reliance on actual human tissue.

With the SCID mouse xenogeneic transplantation model, investigators have studied the relative contributions of various components of the immune system on the etiology and pathophysiology of psoriasis. Nickoloff, *supra*, reported the validity of the SCID mouse animal model system in 1995 and disclosed its utility in studies designed to decipher the mechanism underlying the genetic and etiological abnormalities associated with psoriasis as well as to illuminate the disease's pathophysiological basis. *Id.* Wrono-Smith, T. et al., further demonstrated the utility of the SCID animal model system in mechanistic studies from which it was reported that psoriasis is mediated by immunocytes derived from the circulation and that activated immunocompetent cells secondarily induce keratinocyte and endothelial cell proliferation. *J. Clin. Invest.*, 98(8):1878-1887 (1996). More recently, Gilhar, A. et al. investigated the role of T lymphocytes in psoriatic pathology using the SCID mouse animal model system, *J. Invest. Derm.*, 109(3):283-288 (1997), noting that skin-infiltrating T lymphocytes, but not T-cells derived from peripheral blood, maintained the psoriatic phenotype of human skin grafted onto SCID mice. And, most recently, Torres, B.A. et al. used the SCID mouse

model to study the role of bacterial and viral superantigens in the progression of psoriasis.

*Cur. Opin. Immunol.*, 10(4):465-470 (1998).

In addition to the SCID mouse animal model system other animal model systems have been described for a variety of transplant rejections. For example, cardiac allografts and xenografts have been achieved in Lewis rats using Lewis and Wistar Furth rats as donors, respectively. Luketich, J.D. et al., *Surgery* 106(2):209-215 (1989). See, also, DiSesa, V.J. et al., *J. Thorac. Cardiovasc. Surg.* 101:446-449 (1991) (reporting on the use of Lewis X Brown-Norway and Lewis rat donors as models for cardiac allografts and isografts, respectively). Similarly, corneal transplants have been reported in a Lewis and Fisher rat model system where the efficacy of, for example, neutralizing antibodies was tested (Yatoh, S. et al., *Transplantation* 66:1519-1524 (1998); see also, Ayliffe, W. et al., *Br. J. Ophthalmol.* 76:602 (1992); and Williams K.A., et al., *Invest. Ophthalmol. Vis. Sci.* 26:23 (1985)) and in a rabbit model system where an immunosuppressive agent was examined (Kobayashi, C. et al., *Transplant Proc.* 21:3156 (1989)).

Reisner described the engraftment of xenogenic cells in normal animals having reconstituted hematopoietic deficient immune systems. U.S. Patent No. 5,866,757. These chimeric mammals may be used in the study of transplant rejection in general, and human transplant rejection in particular, as an animal model suitable for the testing of potential drugs and therapies such as the CD40 antagonist gene delivery vectors of the present invention.

An alternative model for transplant rejection is the murine popliteal lymph node (PLN) assay, a graft vs. host model that predicts activity of compounds in blocking human transplant rejection and that has been used routinely to evaluate compounds that are in use clinically. For example, cyclosporin and cyclophosphamide are both active in the murine PLN animal model system and are both used clinically. Morris et al., *Transplantation Proceedings* 22(Sup. 1):110-112 (1990).

It will be apparent that alternative animal model systems for autoimmune diseases and transplant rejections readily available in the art may be preferred depending on the particular gene delivery vector system employed or the particular disease being treated. Thus, the present invention is not limited to the specific exemplary animal models disclosed herein.

Administration of Gene Delivery Vectors Comprising  
CD40 Antagonist Polynucleotides

As provided herein, the compositions for and methods of treating  
5 autoimmune diseases and transplant rejections may utilize one or more gene delivery  
vector used singularly or in combination with other therapeutics to achieve the desired  
diminution of the autoimmune disease or transplant rejection of interest.

- 10 i. The gene delivery vectors of the present invention may be  
administered by intravenous delivery or by other methods  
described herein in order to induce long term *in vivo*  
expression of a variety of CD40 antagonists.

Administration of a retroviral, adenoviral, adeno-associated viral, herpes  
viral or other gene delivery vector comprising a CD40 antagonist polynucleotide  
15 intravenously results in sustained long-term systemic expression of therapeutic proteins  
or antibodies. Thus, methods for obtaining long-term systemic expression *in vivo* of a  
variety of CD40 antagonists is encompassed by the instant invention. For long-term  
expression from a retroviral vector *in vivo*, the action of human complement on the  
retroviral vector is suppressed. This can be achieved by a number of techniques known  
20 to those skilled in the art. Preferably, as indicated, *supra*, human packaging cell lines  
are used in order to inhibit the action of human complement on the retroviral vector  
particles.

The present invention provides the production of high titer preparations  
of, for example, recombinant retroviral or adeno-associated viral vectors. The phrase  
25 "high titer viral vector preparation" as used herein refers to a viral vector preparation  
that has a titer greater than  $10^6$  cfu/ml, more preferably greater than  $10^7$  cfu/ml, still  
more preferably greater than  $10^8$  cfu/ml, more preferably greater than  $10^9$  cfu/ml, yet  
more preferably greater than  $10^{10}$  cfu/ml, and most preferably greater than  $10^{11}$  cfu/ml.  
The level of expression will vary depending on the packaging cell line employed, *e.g.*,  
30 human HT1080 cells for retroviral packaging. The term "cfu" refers to colony forming  
units when vectors contain a selectable marker. The term refers to identifiable colonies  
when a phenotypically observable marker is used, such as blue colonies when the  
marker is beta-galactosidase. When no marker is used, "cfu" may be determined, for

example, by PCR-based titer. Thus, packaging cells are transduced with the markerless vector and the number of proviral DNA copies are measured by quantitative PCR.

The phrases "long term systemic expression" or "sustained systemic expression" as used herein in reference to *in vivo* expression of a CD40 antagonist encoded by a gene delivery vector mean measurable or biologically active expression into the bloodstream for 30 days, more preferably for 60 days, yet more preferably for 90 days, more preferably for six months, still more preferably for 1 year and most preferably for at least 5 years after administration of the gene delivery vector to a host. Expression may be achieved for the life of the patient either by a single administration or by one or more additional "booster" injections. By "systemic expression" is meant that CD40 antagonist proteins are expressed into the circulation and are thus useful for treatment of certain diseases. Expression levels may be measured, for example, by an ELISA assay specific for the given CD40 antagonist administered by the gene delivery vector system. As discussed, *supra*, a number of autoimmune diseases or transplant rejections are amenable to treatment by this type of gene delivery vector system.

It may be advantageous to administer CD40 antagonists of the present invention in conjunction with additional co-stimulatory blockade molecules. For example, in the case of transplant rejections, depending on the particular transplant regimen envisioned, increased therapeutic efficacy including long-term graft survival may be enhanced by co-administration of a CD40 antagonist gene delivery vector in conjunction with transfusion of donor-specific lymphocytes or CTLA4Ig. For further guidance, *see, e.g.*, Sayegh, M. et al., *Transplantation* 64:1646-1650 (1997) and Larsen, C.P. et al., *Nature (Lond.)* 381:434-438 (1996). More specifically, studies of islet and renal allograft transplants in nonhuman primates suggest that CTLA4Ig administration alone may be inadequate therapeutically, but that the combination of CTLA4Ig and antiCD40L antibody together have an additive effect. Kirk, A.D. et al., *Proc. Natl. Acad. Sci. U.S.A.* 94:8789-8794 (1997); Levisetti, M. et al., *J. Immunol.* 159:5187-5191 (1997); and Chang, A.C. et al., *Proc. 23<sup>rd</sup> Am. Soc. Transplant Surgeons Ann. Meeting*, Thorofare, N.J., *American Society of Transplant Surgeons* (1997).

Having identified more than one CD40 antagonist that is effective in an animal model, it may be further advantageous to mix two or more gene delivery vectors encoding distinct CD40 antagonists together to provide still improved efficacy against

autoimmune diseases or transplant rejections. Compositions comprising one or more CD40 antagonist gene delivery vector may be administered to persons or mammals suffering from, or predisposed to suffer from, an autoimmune disease or transplant rejection. CD40 antagonists are believed to minimize the severity of autoimmune diseases by reducing the infiltration of target cells with inflammatory lymphoid cells such as mononuclear phagocytes, lymphocytes, plasma cells and secondary lymphoid follicles and, in the specific case of psoriasis, by diminishing the severity of akathosis and parakeratosis.

By the present methods, compositions comprising CD40 antagonist gene delivery vectors may be administered parenterally, topically, orally or locally for therapeutic treatment. Preferably, the compositions are administered orally or parenterally, *i.e.*, intravenously, intraperitoneally, intradermally or intramuscularly. Thus, this invention provides methods which employ compositions for administration which comprise one or more CD40 antagonists in a pharmaceutically acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be used, *e.g.*, water, buffered water, 0.4% saline, 0.3% glycine and the like, and may include other proteins for enhanced stability, such as albumin, lipoprotein, globulin, etc., subjected to mild chemical modifications or the like. Preferred CD40 antagonists will also exhibit minimal toxicity when administered to a mammal afflicted with an autoimmune disease.

The compositions of the invention may be sterilized by conventional, well known sterilization techniques. The resulting solutions may be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride and stabilizers (*e.g.*, 1-20% maltose, etc.).

The CD40 antagonist gene delivery vectors of the present invention may also be administered via liposomes. Liposomes, which include emulsions, foams, micelles, insoluble monolayers, phospholipid dispersions, lamellar layers and the like, can target the CD40 antagonist gene delivery vectors to a particular tissue as well as

increase the half-life of the composition. A variety of methods are available for preparing liposomes, as described in, e.g., U.S. Patent Nos. 4,837,028 and 5,019,369, which patents are incorporated herein by reference. Actual methods for preparing orally, topically and parenterally administrable compositions will be known or apparent to those skilled in the art and are described in detail in, for example, *Remington's Pharmaceutical Science*, 19<sup>th</sup> ed., Mack Publishing Co., Easton, PA (1995), which is incorporated herein by reference.

Determination of an effective amount of a composition of the invention to treat an autoimmune disease or transplant rejection in a patient can be accomplished through standard empirical methods which are well known in the art. For example, in the case of psoriasis, reversal of akathosis and parakeratosis as well as diminution in lymphocyte infiltration in the keratinocytes can be measured.

Compositions of the invention are administered to a mammal already suffering from an autoimmune disease or predisposed to an autoimmune disease in an amount sufficient to prevent or at least partially arrest the development of the autoimmune disease. Similarly, compositions for the treatment of transplant rejections may be administered in such a dose so as to reduce or eliminate the onset of graft rejection. An amount adequate to accomplish this is defined as a "therapeutically effective dose." Effective amounts of a gene delivery vector will vary and depend on the severity of the disease and the weight and general state of the patient being treated. Administration is daily, weekly or less frequently, as necessary depending on the response to the disease and the patient's tolerance of the therapy. Maintenance dosages over a prolonged period of time may be needed, and dosages may be adjusted as necessary.

Single or multiple administrations of the compositions can be carried out with the dose levels and pattern being selected by the treating physician. In any event, the formulations should provide a quantity of CD40 antagonist gene delivery vector sufficient to effectively prevent or minimize the severity of the autoimmune disease or transplant rejection. The compositions of the present invention may be administered alone or as an adjunct therapy in conjunction with other therapeutics well known in the art for the treatment of autoimmune diseases or transplant rejections.

The methods of the invention can also be employed for *ex vivo* or extracorporeal therapy against autoimmune diseases by performing the therapeutic manipulations on peripheral blood mononuclear cells (PBMC) outside of the body. For example, PBMC may be removed from the subject and treated with the inventive gene  
5 delivery vector. These cells may be subsequently administered to the subject to block or substantially reduce the activation of CD4<sup>+</sup> T-cells. By performing the administration of the gene delivery vector outside of the subject's body, significantly higher concentrations of the gene delivery vector may be employed than would be tolerated through *in vivo* administration. *Ex vivo* applications of the present methods  
10 may further comprise the administration of additional agents which together provide enhanced therapeutic activity against autoimmune diseases and transplant rejections.

The compositions of the present invention also find use *in vitro*. For example, gene delivery vectors encoding CD40 antagonists may be used to inhibit superantigen activation of PBMCs *in vitro*. Alternatively, inventive compositions can  
15 be used in screening assays to assess the effective levels of therapeutics or other treatments for autoimmune diseases and transplant rejections. In other embodiments, the present compositions may be used in the design or screening of various potential treatment modalities, such as methods for the treatment of psoriasis or other autoimmune disease as well as transplant rejections. Thus, a diagnostic method for  
20 assessing the efficacy of, *e.g.*, autoimmune therapeutics is also provided by the present invention. Detecting changes *in vitro* which parallel the reversal of an autoimmune disease, for example, the diminution of CD25 and CD69 levels in CD4<sup>+</sup> T-cells following bacterial superantigen administration to PBMC, provides an indication of *in vivo* activity of the CD40 antagonist intended for treatment in accordance with the  
25 present invention.

### EXAMPLES

The following experimental examples are offered by way of illustration, not limitation.

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#### EXAMPLE 1

##### CONSTRUCTION OF THE RECOMBINANT AAV GENE DELIVERY VECTOR D10p $\alpha$ CD40-1 FOR THE GENERATION OF A SINGLE CHAIN ANTIBODY AGAINST CD40

The D10-1 AAV vector, *see* Figure 1, was constructed by replacing the AAV gene encoding sequences of pD-10, *see* Wang, X. et al., *J. Virol.* 71:3077 (1997),  
10 incorporated herein by reference, with the 5' and 3' AAV-2 inverted terminal repeats (ITRs), CMV immediate early promoter/enhancer, multiple cloning site and bovine growth hormone (BGH) polyadenylation sequences from pKm201CMV.

pKm201CMV is an AAV cloning vector in which an expression cassette, consisting of a CMV immediate early promoter/enhancer and a bovine growth hormone  
15 (BGH) polyadenylation site, is flanked by inverted terminal repeat (ITR) sequences from AAV-2. Briefly, pKm201CMV was derived from pKm201, a modified AAV vector plasmid in which the ampicillin resistance gene of pEMBL-AAV-ITR (*see* Srivastava (1989) *Proc. Natl. Acad. Sci. USA* 86:8078-8082) had been replaced with the gene for kanamycin resistance. The expression cassette from pCMVlink, a  
20 derivative of pCMV6c (*see* Chapman (1991) *Nucleic Acids Res.* 19:193-198) in which the BGH poly A site has been substituted for the SV40 terminator, was inserted between ITRs of pKm201 to generate pKm201CMV.

D10p $\alpha$ CD40-1 was derived from D10-1 by inserting a CD40-scFv cassette at the Hind III and XbaI sites of the D10-1 multiple cloning site. In addition, a  
25 1.35 kb  $\phi$ X174 HaeIII restriction fragment was ligated between the 5' ITR and CMV enhancer/promoter sequence, at the NotI site, to increase the size of the resulting plasmid in order to increase the sequence length between the 5' and 3' ITRs so as to optimize viral packaging.

The CD40-scFv cassette encodes a fusion protein comprised of an anti-  
30 CD40 antagonist antibody heavy chain and light chain Fab sequence of antibody 5H7. The hybridoma cell line 5H7 produces a humanized mouse monoclonal antibody which binds CD40 and exhibits a CD40 antagonist activity. 5H7 is effective in inhibiting the

manifestations of autoimmune diseases such as psoriasis and is described in detail in co-pending application no. 60/157,461.

RNA was isolated from the 5H7 hybridoma and used as template in a reverse transcription-polymerase chain reaction (RT-PCR) using a pair of primers that hybridize to the corresponding heavy and light chain Fab coding regions. These primers have the nucleotide sequence as indicated in SEQ ID NO: 3, SEQ ID NO:4, SEQ ID NO:5 and SEQ ID NO:6. Amplified cDNA fragments of the heavy and light variable regions were connected by a polynucleotide linker. The resulting fusion construct was cloned into the pBS-KS plasmid vector and subcloned into the D10-1 AAV gene delivery vector to create D10p $\alpha$ CD40-1 as described above.

## EXAMPLE 2

### PRODUCTION OF THE SINGLE CHAIN FV HEAVY AND LIGHT CHAIN ANTI-CD40

#### ANTAGONIST FUSION PROTEIN EXPRESSED FROM THE D10p $\alpha$ CD40-1

#### 15 GENE DELIVERY VECTOR

Expression of the CD40 antagonist expressed in the D10p $\alpha$ CD40-1 gene delivery vector system was confirmed by competition assay whereby the CD40 antagonist blocks the binding of the native 5H7 antibody to CD40 expressing cells. Specifically, D10p $\alpha$ CD40-1 and D10-1 were transiently transfected into COS-cells and culture supernatants were collected after 36 hours growth at 37°C in Dulbecco's modified eagle's medium (DMEM).

Ramos-S cells, which cells express CD-40, were used to assess the anti-CD40 binding activity of the COS-cell culture supernatants.  $1 \times 10^5$  Ramos-S cells were pre-blocked on ice with FcR prior to incubating with either D10-1 or D10p $\alpha$ CD40-1 transfected COS-cell supernatant. After removing unbound supernatant, the Ramos-S cells were subsequently incubated in the presence of either culture medium or 5H7 antibody. Binding of 5H7 to CD40 was assessed by complex formation with FITC conjugated anti-mouse-IgG2b (available from Pharmagen, San Diego, CA), with detection by fluorescence activated cell sorting (FACS). The results of a representative experiment is depicted in Table 1. These data show that the CD40 antagonist expressed off the D10paCD40-1 vector is effective in binding to CD40

expressed on Ramos-S cells and, thereby, in blocking subsequent interaction of the CD40 receptor with the 5H7 anti-CD40 antibody.

TABLE 1

	Ramos-S	D10-1 (LT-1)	D10p□ CD40-1/ G7P (LT-1)	D10pa CD40-1 (LT-1)	D10-1 (Lipo)	D10p□ CD40-1/ G7P (Lipo)	D10pa CD40-1 (Lipo)	5H7 (Lipo 1:4.5)	Total	X Mean	Median
1	+	-	-	-	-	-	-	-	89.77	9.7	8.43
2	+	-	-	-	-	-	-	-	89.82	13.16	11.86
3	+	+	-	-	-	-	-	-	88.33	12.59	11.04
4	+	-	+	-	-	-	-	-	89.56	16.95	11.24
5	+	-	-	+	-	-	-	-	87.87	12.97	11.34
6	+	-	-	-	+	-	-	-	88.86	12.87	11.34
7	+	-	-	-	-	+	-	-	89.48	14.68	11.44
8	+	-	-	-	-	-	+	-	89.12	13.06	11.44
9	+	-	-	-	-	-	-	-	90.21	13.95	11.55
10	+	-	-	-	-	-	-	+	90.07	59.03	55.23
11	+	+	-	-	-	-	-	+	89.83	52.74	49.14
12	+	-	+	-	-	-	-	+	87.85	30.75	25.48
13	+	-	-	+	-	-	-	+	85.27	27.46	25.48
14	+	-	-	-	+	-	-	+	89.53	53.23	49.58
15	+	-	-	-	-	+	-	+	89.68	26.79	23.29
16	+	-	-	-	-	-	+	+	88.99	24.45	22.88
17	+	-	-	-	-	-	-	+	88.94	26.95	25.48

From the foregoing it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not to be limited except as by the appended claims.

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CLAIMS

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What is claimed is:

1. An isolated polynucleotide encoding a CD40 antagonist.
2. The isolated polynucleotide of claim 1 wherein said CD40 antagonist is an antibody or a fragment thereof.
3. The isolated polynucleotide of claim 1 wherein said CD40 antagonist is a fusion protein.
4. The isolated polynucleotide of claim 3 wherein said fusion protein comprises a portion of at least one anti-CD40 antibody Fv heavy chain and a portion of at least one anti-CD40 antibody Fv light chain.
5. The isolated polynucleotide of claim 1 comprising a polynucleotide sequence that is at least 70% identical with the polynucleotide sequence of SEQ ID NO:1.
6. The isolated polynucleotide of claim 1 comprising a polynucleotide or the complement thereof that hybridizes under stringent conditions to the polynucleotide of SEQ ID NO:1.
7. A gene delivery vector, comprising a polynucleotide that encodes a CD40 antagonist.
8. The gene delivery vector of claim 7, further comprising transcriptional regulatory sequences of a virus selected from the group consisting of a retrovirus, an adenovirus, an adeno-associated virus and a herpes virus.

9. The gene delivery vector of claim 7, further comprising an adeno-associated virus 5' inverted terminal repeat (ITR), a CMV immediate early enhancer/promoter and a 3' ITR.

10. The gene delivery vector of claim 9, further comprising a bovine growth hormone (BGH) polyadenylation sequence.

11. The gene delivery vector of claim 7 wherein said CD40 antagonist is capable of specifically binding to CD40.

12. The gene delivery vector of claim 7 wherein said CD40 antagonist is an antibody or a fragment thereof.

13. The gene delivery vector of claim 7 wherein said CD40 antagonist is a monoclonal antibody or a fragment thereof.

14. The gene delivery vector of claim 7 wherein said CD40 antagonist is a fusion protein, wherein said fusion protein comprises a portion of at least one anti-CD40 antibody Fv heavy chain and a portion of at least one anti-CD40 antibody Fv light chain.

15. The gene delivery vector of claim 7 wherein said polynucleotide is at least 70% identical to the nucleotide sequence of SEQ ID NO:1.

16. The gene delivery vector of claim 7 wherein said polynucleotide comprises the nucleotide sequence of SEQ ID NO:1.

17. The gene delivery vector of claim 7, wherein said polynucleotide or complement thereof hybridizes under stringent conditions to the nucleotide sequence of SEQ ID NO:1.

18. The gene delivery vector of claim 7 comprising the polynucleotide sequence of D10 $\alpha$ CD40-1.

19. A composition for treating an autoimmune disease, comprising a therapeutically effective amount of the gene delivery vector of claim 10 and a pharmaceutically acceptable carrier.

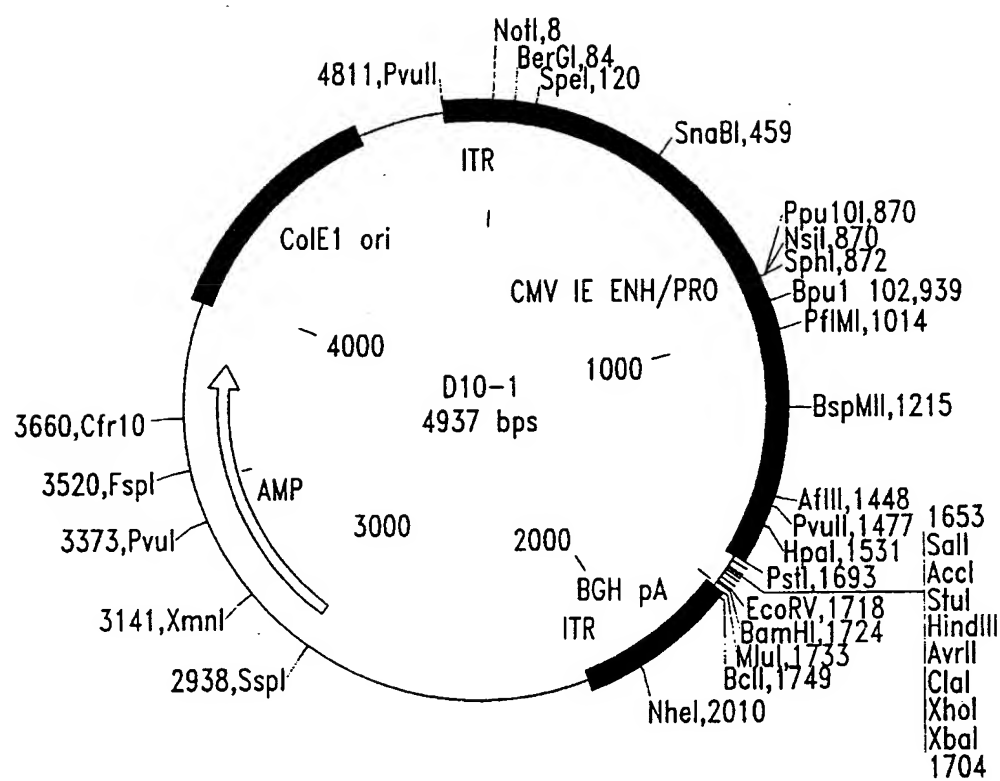
20. The composition of claim 19 wherein said autoimmune disease is selected from the group consisting of Hashimoto's thyroiditis, primary myxoedema thyrotoxicosis, pernicious anemia, Addison's disease, insulin-dependent diabetes mellitus, systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis, dermatomyositis, scleroderma and psoriasis.

21. The composition of claim 19, wherein said autoimmune disease is psoriasis.

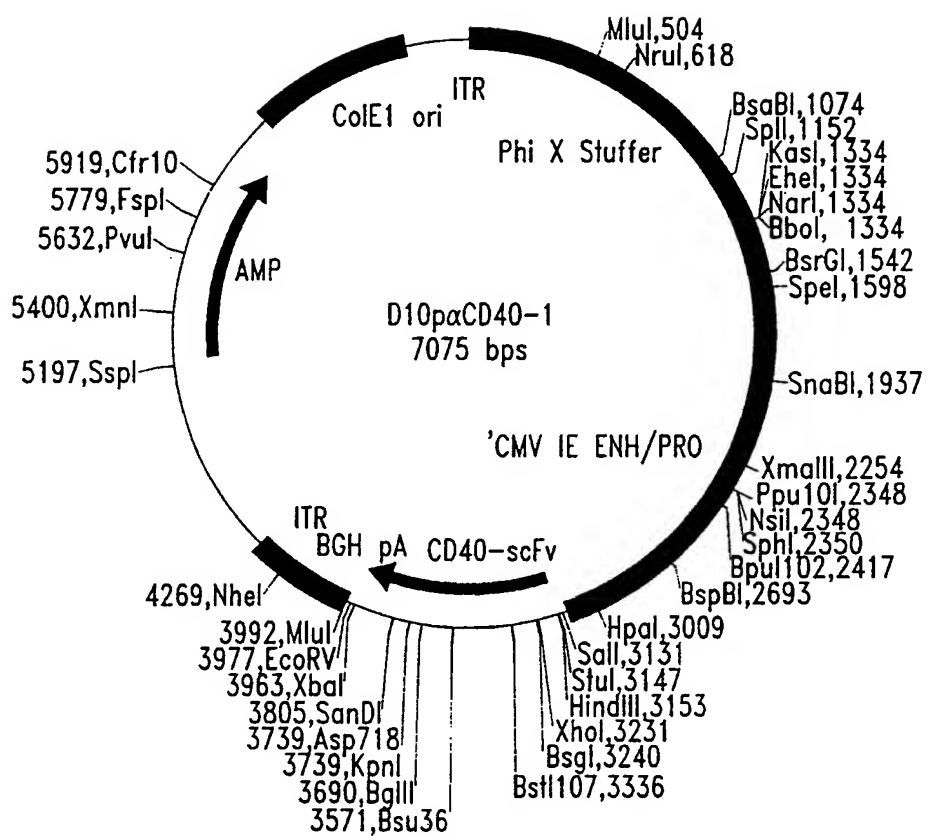
22. A composition for treating a transplant rejection, comprising a therapeutically effective amount of the gene delivery vector of claim 7 and a pharmaceutically acceptable carrier.

23. The composition of claim 22 wherein said transplant rejection is of cardiac, hepatic, islet, renal, lung and bone marrow tissue.

1/2

*Fig. 1*

2/2

*Fig. 2*

## SEQUENCE LISTING

<110> Chiron Corporation  
 Chu, Keting  
 Wang, Changyu

<120> COMPOSITIONS AND METHODS FOR TREATING  
 AUTOIMMUNE DISEASE AND TRANSPLANT REJECTIONS

<130> 200130.496/PP01528.003

<140> PCT

<141> 2000-11-08

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Ser	Arg	Leu	Ser	Ile	Ser	Lys	Asp	Thr	Ser	Lys	Ser	Gln	Val	Phe	Leu	
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Lys	Met	Asn	Ser	Leu	Gln	Thr	Asp	Asp	Thr	Ala	Met	Tyr	Tyr	Cys	Val	
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Pro Lys Leu Leu Ile Tyr Lys Val Ser Asn Arg Phe Ser Gly Val Pro			
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24

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